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The Mediation of Platelet Quiescence by NO-Releasing Polymers via cGMP-Induced Serine 239 Phosphorylation of Vasodilator-Stimulated Phosphoprotein

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Abstract

Nitric oxide (NO) releasing (NORel) materials have been shown to create localized increases in NO concentration by the release of NO from a diazeniumdiolate-containing or *S*-nitrosothiol-containing polymer coating and the improvement of extracorporeal circulation (ECC) hemocompatibility. However, the mechanism and, in particular, the platelet upregulation of the NO/cGMP signaling protein, vasodilator-stimulated phosphoprotein phosphorylated at serine 239 (P-VASP (ser 239), for the improved ECC hemocompatibility via NO release still needs elucidation. In this work, two NORel polymeric coatings were evaluated in a 4 h rabbit thrombogenicity (RT) model and the anti-thrombotic mechanism investigated for rabbit platelet P-VASP upregulation. Polymer films containing 25 wt% diazeniumdiolated dibutylhexansdiamine (DBHD) or 5 wt% *S*-nitroso-*N*-acetylpenicillamine (SNAP) coated on the inner walls of ECC circuits yielded significantly reduced ECC thrombus formation and maintained normal platelet

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aggregation compared to polymer controls after 4 h of blood exposure. Platelet P-VASP (ser 239), a useful tool to monitor NO/cGMP signaling, was upregulated after 4 h on ECC and markedly increased after ex vivo sodium nitroprusside (SNP) stimulation. Interestingly, in the rabbit platelet, NO did not upregulate the cAMP P-VASP phosphoprotein P-VASP (ser 157) as previously shown in human platelets. These results suggest that NOrel polymers preserve rabbit platelet quiescence by sustaining a level of cGMP signaling as monitored by P-VASP (ser 239) upregulation. The upregulation of this NO-mediated platelet signaling mechanism in this RT model indicates the potential for improved thromboresistance of any NOrel-coated medical device.

Keywords

nitric oxide (NO); hemocompatibility; platelets; extracorporeal circulation (ECC); vasodilator-stimulated phosphoprotein (VASP); cGMP

1. Introduction

The quiescence of circulating blood platelets as blood passes through polymeric tubing used in extracorporeal life support (ECLS) has been the elusive goal for hemocompatible polymer research for several years. Systemic anticoagulation, particularly with heparin, has allowed ECLS to be of significant success over the past decades but not without the possible adverse effect of bleeding risk. Within the last 20 years of research, agents immobilized heparin or nitric oxide (NO) donors have been used as ECLS surface coatings to provide local anticoagulation without the systemic adverse effects [1-5]. Nitric oxide, an endogenous platelet inhibitor and vasodilator, has been incorporated into polymeric tubing to mimic its endogenous inhibitory effects on platelet activation and, thus, attenuate extracorporeal circuit (ECC) thrombus formation. A number of NO releasing (NOrel) polymer coatings have been studied over the last decade using polymer-embedded NO donor molecules, such as diazeniumdiolated dibutylhexanediamine (DBHD/N₂O₂) [1, 6] and *S*-nitrosothiols including *S*-nitroso-*N*-acetylpenicillamine (SNAP) [7, 8]. These two polymeric coatings release NO by distinctly different processes but still preserve platelet quiescence and maintain their normal function systemically. Even though differences in the local release of NO in the two polymers exist, the underlying question is if the released NO preserves platelet quiescence through a similar mechanism of upregulation of the NO/cGMP signaling pathway and resultant inhibition of platelet activation.

NO, released from vascular endothelial cells, regulates the cellular NO/cGMP signaling pathway and the resultant vasodilation in smooth muscle cells [9, 10] or inhibition of platelet activation [11-13]. NO mediates these cellular events by directly activating its target enzyme soluble guanyl cyclase (sGC) and increasing the cytosolic levels of the second messenger cGMP [14]. Increases in intracellular cGMP elicit an upregulation of the cGMP-dependent protein kinase types I and II (cGKI and II) of which cGKI lowers the intracellular level of cytosolic calcium [15, 16] and is therefore considered important for the inhibition of platelet activation. The substrate for cGKI in many cells is the 46-kDa/50-kDa vasodilator-stimulated phosphoprotein (VASP) which was first discovered as a substrate for both the cAMP and cGMP signaling pathways and when phosphorylated by activated cGKI or cAK (cAMP-dependent protein kinase) causes the focal adhesion of cytoskeletal filaments, namely actin, into spatial confinement preventing platelet shape change upon activation [17-19]. The phosphorylation of VASP in human platelets has been shown to occur at two major sites, serine 157 and serine 239 [20]. Phosphorylation of the serine 157 and serine 239 sites have preferentially been activated by cAK and cGKI, respectively, in human platelets [17]. However, in rabbit platelets, the phosphorylation of serine 157 or serine 239 has not been determined when stimulated by cAK or cGK agonists such as prostaglandin E1 (PGE1)

or NO. With NOrel polymers releasing NO locally through different processes, one asks if the released NO mediates the cellular NO/cGMP pathway similarly. The mechanism of this polymer-released NO has not been studied for its effects on P-VASP (ser 157 or 239) in circulating platelets, and particularly, in platelets from the rabbit thrombogenicity (RT) model.

This work, therefore, investigated the possible mechanism of NO released locally from different NOrel polymeric coatings on reducing ECC thrombus formation and preservation of platelet function after 4 h blood exposure to ECC in the RT model. If NO released from these polymers attenuate rabbit platelet activation via the upregulation of the NO/cGMP signaling pathway, phosphorylation of the cGMP protein kinase-dependent vasodilator-stimulated phosphoprotein (VASP) should be increased when stimulated by NO.

2. Materials and Methods

2.1. Materials

Tygon® poly(vinyl chloride) (PVC) tubing was purchased from Fisher Healthcare (Houston, TX). Elast-eon™ (E2As) was obtained from AorTech International, plc (Scoresby, Victoria, Australia). Tetrahydrofuran (THF) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). S-nitroso-N-acetyl-DL-penicillamine (SNAP) was prepared from N-Acetyl-DL-penicillamine (NAP), which was obtained from Sigma-Aldrich (St. Louis, MO), by reacting for 30 min NAP with equimolar sodium nitrite in a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H₂SO₄. The mouse antibodies for human CD61 (GPIIIa) FITC and human P-selectin glycoprotein (CD62P) PE were from AbD Serotec (Raleigh, NC) as well as Mouse isotype controls for IgG₁ FITC and IgG₁ PE. The antibody clone for P-selectin has been previously shown to have cross-reactivity to the rabbit [16-19]. For western blotting, antibodies used for P-VASP (ser157), P-VASP (ser 239), cGK1, P-Selectin and VASP were FITC-conjugated mouse anti-human P-VASP (ser 157) (5C6), FITC-conjugated mouse anti-human P-VASP (ser 239) (16C2), goat anti-human cGK1 (L-16), rabbit anti-human P-Selectin (C-20) and goat anti-human VASP (H-90), respectively. All other reagents were of the highest purity available.

2.2. Preparation of Diazeniumdiolated Polymeric Coating and ECC Construction

The DBHD/N₂O₂ and PLGA polymer coating solution containing 25 wt% DBHD/N₂O₂ and 10 wt% PLGA in Elast-eon™ (E2As) polyurethane. Briefly, triple layers of polymeric coatings which included an active layer and a top layer were individually coated on the inner wall of the Tygon® tubing that formed the ECC. The active layer was prepared by dissolving 2250 mg E2As and 180 mg PLGA in 10 mL THF (Soln A). Four hundred and fifty milligrams DBHD/N₂O₂ was then dispersed within the polymer cocktail by sonication for 30 min to obtain a slightly cloudy dispersion of the diazeniumdiolate in the solution (Soln B). The top coat solution was prepared using 2250 mg E2As in 10 mL THF (Soln C).

The ECC configuration used in this study was previously described.[6] The ECC consisted of a 16-gauge and 14-gauge IV polyurethane angi catheters (Kendall Monoject Tyco Healthcare Mansfield, MA), two 16 cm lengths of 1/4 inch inner diameter (ID) Tygon® tubing and a 8 cm length of 3/8 inch ID Tygon® tubing which created a thrombogenicity chamber where thrombus could form more easily due to more turbulent blood flow. The ECC was pieced together, starting at the left carotid artery side, with the 16-gauge angi catheter, one 15 cm length 1/4 inch ID tubing, the 8 cm length thrombogenicity chamber, the second 15 cm length 1/4 inch ID tubing and finally the 14-gauge angi catheter. The angi catheters were interfaced with tubing using 2 luer-lock PVC connectors. The 3/8 inch ID tubing and the 1/4 inch tubing were welded together using THF.

The assembled DBHD/N₂O₂/PLGA ECC was coated first with one base coats of Soln A, then two active or control coats of Soln B, followed by one top coat of Soln C. Each coat was allowed to dry for at least 1 h. The finished ECCs were allowed to cure under nitrogen conditions for 20 min, and then dried under vacuum for 2 d (Figure 1A).

2.3. Preparation of RSNO Polymeric Coating and ECC Construction

SNAP was synthesized using a modified version of a previously reported method [21]. Briefly, an equimolar ratio of NAP and sodium nitrite was added to a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H₂SO₄. After 30 min of stirring, the reaction vessel was placed in an ice bath, and a green precipitate formed. The crystals were collected by filtration, washed with water, and allowed to air dry. The reaction and crystals were protected from light at all times.

The 5 wt% SNAP/E2As solution was prepared by dissolving 125 mg SNAP and 2250 mg E2As in 15 mL of THF. The E2As control solution consisted of 2500 mg E2As in 15 mL THF. SNAP/E2As loops were first coated with 2 coats of the SNAP/E2As solution, followed by 1 coat of the E2As control solution. E2As control loops were coated with 2 coats of the E2As control solution. ECC loops were allowed to air dry for 1 h in the dark between each coat. The completely coated ECC was welded together using THF, starting at the left carotid artery side, with the 16-gauge angiocatheter, one 15 cm length 1/4 inch ID tubing, the 8 cm length thrombogenicity chamber, the second 15 cm length 1/4 inch ID tubing and finally the 14-gauge angiocatheter. The angiocatheters were interfaced with tubing using two luer-lock PVC connectors. The assembled ECC loops were dried under vacuum while protected from light for at least 48 h (Figure 1B). Prior to the ECC experiments, the loops were filled with saline solution for overnight soaking, and this solution was discarded immediately before the rabbit experiment.

2.4. NO Release Measurements

NO released from the two NOrel polymer-coated circuits was measured via a Sievers chemiluminescence NO Analyzer® (NOA), model 280 (Boulder, CO) as described previously [6]. Briefly, a 5 min measurement of baseline NO level was obtained and then a centimeter square sample of the DBHD/N₂O₂- or SNAP-containing circuitry was then placed in the reaction vessel containing PBS buffer at 37° C. NO was continuously swept from the headspace of the sample vessel and purged from the bathing solution with a nitrogen sweep gas and bubbler into the chemiluminescence detection chamber. The flow rate was set to 200 ml/min with a chamber pressure of 5.4 Torr and an oxygen pressure of 6.0 psi. Using this method, a uniform segment of either polymer was tested for NO release prior to and 4 h after blood exposure in the rabbit thrombogenicity model.

2.5. The Rabbit Thrombogenicity Model

The animal handling and surgical procedures were approved by the University Committee on the Use and Care of Animals in accordance with university and federal regulations. A total of 22 Zealand white rabbits (Covance, Inc. Battle Creek, MI) were used in this study. All rabbits (2.5-3.5 kg) were initially anesthetized with intramuscular injections of 5 mg/kg xylazine injectable (AnaSed® Lloyd Laboratories Shenandoah, Iowa) and 30 mg/kg ketamine hydrochloride (Hospira, Inc. Lake Forest, IL). Maintenance anesthesia was administered at a rate of 2-4 % via an isoflurane inhalation gas anesthesia machine and an ADS 2000 small animal ventilator (Engler Engineering Corp. Hialeah, FL). In order to maintain blood pressure stability, IV fluids of Lactated Ringer's were given at a rate of 10 ml/kg/h. For monitoring blood pressure and collecting blood samples, the rabbits' right carotid artery was cannulated using a 16-gauge IV angiocatheter (Jelco®, Johnson & Johnson, Cincinnati, OH). Blood pressure and derived heart rate were monitored with a

Series 7000 Monitor (Marquette Electronics Milwaukee, WI). Body temperature was monitored with a rectal probe and maintained at 37°C using a water-jacketed heating blanket. Prior to placement of the arteriovenous (AV) custom-built extracorporeal circuit (ECC), the rabbit left carotid artery and right external jugular vein were isolated and baseline hemodynamics as well as arterial blood pH, pCO₂, pO₂ and total hemoglobin were measured using an ABL 725 blood-gas analyzer (Radiometer Copenhagen Copenhagen, DK). In addition, baseline blood samples were collected for platelet counts which were measured on a Coulter Counter Z1 (Coulter Electronics Hialeah, FL). Plasma fibrinogen levels were determined using a Dade Behring BCS Coagulation Analyzer (Siemens Deerfield, IL), activated clotting times (ACT) were monitored using a Hemochron Blood Coagulation System Model 801 (International Technidyne Corp. Edison, NJ), platelet function was assessed using a Chrono-Log optical aggregometer model 490 (Havertown, PA) and platelet P-selectin expression were determined utilizing fluorescent-activated cell sorting (FACS) with a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA).

After baseline blood measurements, the AV custom-built ECC was placed into position by cannulating the left carotid artery for ECC inflow and the right external jugular vein for ECC outflow. The flow through the ECC was started by unclamping the arterial and venous sides of ECC and blood flow in circuit was monitored with an ultrasonic flow probe and flow meter (Transonic HT207 Ithaca, NY). Animals were not systemically anticoagulated during the experiments. After four hours on ECC, the circuits were clamped, removed from animal, rinsed with 60 ml of saline and drained. Any residual thrombus in the larger tubing of ECC (i.e., thrombogenicity chamber) was photographed and the degree of thrombus image was quantitated using Image J imaging software from National Institutes of Health (Bethesda, MD). Prior to euthanasia, all animals received a dose of 400 U/kg sodium heparin to prevent necrotic thrombosis. The animals were euthanized using a dose of Fatal Plus (130 mg/kg sodium pentobarbital) (Vortech Pharmaceuticals Dearborn, MI).

2.6. Blood Sampling

Rabbit whole blood samples were collected in non-anticoagulated 1 cc syringes for ACT, 10% anticoagulant containing 3.2% sodium citrate in two 3 cc syringes for cell counts, aggregometry, FACS analysis and western blotting, and 1 cc syringes containing 40 U/ml of sodium heparin (APP Pharmaceuticals, LLC Schaumburg, IL) for blood-gas analysis. Following the initiation of ECC blood flow, blood samples were collected every hour for 4 h for *in vitro* measurements. Samples were used within 2 h of collection to avoid any activation of platelets or plasma fibrinogen.

2.7. Rabbit Washed Platelet Preparation

PRP was prepared from whole rabbit blood by centrifugation at $110 \times g$ for 15 min and removing the plasma to a separate 50 ml centrifuge tube. The PRP was counted on a Beckman Coulter Z1 particle counter and diluted with Hank's Balanced Salt Solution (HBSS; $-Ca^{2+}$, $-Mg^{2+}$) containing 0.32% citrate. A washed platelet pellet from the diluted PRP was obtained by further centrifugation of the PRP at $2730 \times g$ for 10 min and then the supernatant discarded. The platelet pellet was resuspended with HBSS ($+Ca^{2+}$, $-Mg^{2+}$) to a normalized platelet count of 1×10^9 platelets/ml for western blotting studies and to 2×10^8 platelets/ml for aggregometry.

2.8. Platelet Aggregometry

Rabbit platelet aggregation was assayed based on the Born's turbidimetric method using a Chrono-Log optical aggregometer as previously described [6]. Briefly, citrated blood (1:10 blood to 3.2% sodium citrate) was collected (6 ml) at baseline and then each hour up to 4 h from the *in vivo* rabbit ECC studies. Platelet-rich plasma (PRP) was obtained by

centrifugation at $110 \times g$ for 15 min. Platelet-poor plasma (PPP) was obtained by another centrifugation of the PRP-removed blood sample at $2730 \times g$ for 15 min and was used as the blank for aggregation. PRP was incubated for 10 min at 37°C and then $25 \mu\text{g/ml}$ collagen (Chrono-PAR #385 Havertown, PA) was added. The percentage of aggregation was determined 3 min after the addition of collagen using Chrono-Log Aggrolink software.

In separate studies normal rabbit washed platelets were investigated for their collagen-induced aggregation responses to NO/cGMP pathway mediators such as ODQ, inhibitor of sGC, and dipyridamole, a PDE V inhibitor which enhances NO effects with or without the NO donor, sodium nitroprusside (SNP). Briefly, the washed platelet pellet was resuspended with HBSS ($+\text{Ca}^{2+}$, $-\text{Mg}^{2+}$) to a normalized platelet count of 2×10^8 platelets/ml.

Aggregation was done by first incubating the normalized platelets with $4 \mu\text{l}$ DMSO, $20 \mu\text{M}$ ODQ or $10 \mu\text{M}$ dipyridamole were incubated for 10 min at 37°C . The NO donor, SNP, at 0, 10 or $100 \mu\text{M}$ or $4 \mu\text{l}$ saline was then added for a further 10 min at 37°C . Collagen at $25 \mu\text{g/ml}$ was added and aggregation recorded. The maximum percentage of aggregation was determined after the addition of collagen using the PAP-4 aggregometer (Bio/Data Corp. Horsham, PA).

2.9. Flow Cytometry

To determine platelet P-selectin (CD62P) and IIb/IIIa (fibrinogen receptor) expression, 100 μl of diluted blood aliquots (1:100 dilution of blood to HBSS without CaCl_2 and MgCl_2) were directly prepared for cell surface staining of P-selectin and IIb/IIIa. In four 12×75 polypropylene tubes containing 100 μl of diluted blood, $40 \mu\text{g/ml}$ collagen ($4 \mu\text{l}$ $1000 \mu\text{g/ml}$) was added to two tubes and $4 \mu\text{l}$ saline was added to the other two tubes. At this point, saturating concentrations ($10 \mu\text{l}$) of monoclonal antihuman IIIa FITC and monoclonal antihuman CD62P PE antibodies were added to one of the collagen and one of the saline treated tubes and incubated for 15 min at room temperature (RT) in the dark. In the other two tubes containing collagen and saline, $10 \mu\text{l}$ each of antimouse IgG₁ FITC and PE were added as nonbinding isotype controls and also incubated for 15 min at RT in the dark. After the antibody incubation step, each tube received 700 μl of freshly prepared 1% formaldehyde buffer (in dPBS) and was stored at 4°C until ready for flow cytometric analysis. A flow cytometer (FACSCalibur, Becton Dickinson San Jose, CA) was used for the acquisition of flow data and the CellQuest software was employed for data analysis. Cell populations were identified for data collection by their forward scatter (FSC) and side scatter (SSC) light profiles. For each sample, 30,000 total events were collected. Fluorescence intensity of immunostaining was quantitated by a histogram log plot analysis. Mean fluorescent intensity (MFI) was expressed as the geometric mean channel fluorescence minus the appropriate isotype control.

2.10. Platelet P-VASP (ser 157), P-VASP (ser 239), cGKI β , P-Selectin and VASP Expression

Platelet rich plasma from the citrated whole rabbit blood collected over the 4 h experimental period for each NO releasing or control polymer coating was used for preparing washed platelets normalized to 1×10^9 platelets/ml. Normalized platelet suspensions were put into 1.5 ml microfuge tubes and incubated at room temperature for 10 min. After this incubation the platelets were then incubated with or without $100 \mu\text{M}$ SNP (10 min incubation). A platelet lysate was prepared using $50 \mu\text{l}$ $3\times$ sample buffer containing the reducing agent, 2-mercaptoethanol and incubated for 5 min at 95°C . Platelet lysates were stored at -70°C with protein concentration determined by RC/DC protein assay (Bio-Rad Hercules, CA). After the -70°C -stored samples were rapidly thawed in a 37°C water bath, an aliquot of $30 \mu\text{g}$ protein were run on a 4-20% sodium dodecyl sulfate-polyacrylamide gel using a Bio-Rad miniblot electrophoresis system (Criterion Hercules, CA). Protein was transferred onto poly(vinyl-difluoride) membranes for 2 h at 1 amp. Membranes were blocked with Tris-

buffered saline containing 0.05% Tween-20 and 3% nonfat dry milk (Bio-Rad) for 1 h at room temperature (RT) followed by an overnight incubation at 4°C with FITC conjugated mouse anti-human P-VASP (ser 157), goat anti-human P-Selectin (C-20) and rabbit anti-human VASP (H-90) antibodies with gentle rocking. The membranes were washed twice for 10 min each at RT and then secondary antibodies donkey anti-goat IgG (H+L) Alexa Fluor 555 and chicken anti-rabbit IgG (H+L) Alexa Fluor 647 were used to label P-Selectin and VASP proteins, respectively, with gentle rocking. The membranes were washed twice for 10 min each and then imaged in a fluorescence imager (Bio-Rad VersaDoc MP4000 Hercules, CA). The fluorescence intensity of the protein bands were quantified by multi-channel densitometry using Quantity One imaging software (Bio-Rad Hercules, CA). The ratio of P-VASP (ser 157) over unphosphorylated VASP expressions was calculated. The data are means $\pm$ SEM. A second blot was prepared as described above except this blot was incubated with FITC conjugated mouse anti-human P-VASP (ser 239), goat anti-human cGKI (L-16) and rabbit anti-human VASP (H-90) antibodies. The ratio of P-VASP (ser 239) over unphosphorylated VASP expressions was calculated.

In separate studies normal rabbit washed platelets were investigated for their expressions of P-VASP (ser 157), P-VASP (ser 239), cGKI, P-Selectin and VASP to NO/cGMP pathway mediators such as ODQ, inhibitor of sGC, and dipyridamole, a PDE V inhibitor which enhances NO effects with or without the NO donor, SNP. Briefly, the washed platelet pellet was resuspended with HBSS (+Ca²⁺, -Mg²⁺) to a normalized platelet count of 1×10^9 platelets/ml. Washed platelets (100 μ l) were first incubated with 1 μ l DMSO, 20 μ M ODQ or 10 μ M dipyridamole were incubated for 10 min at RT. The NO donor, SNP, at 0, 10 or 100 μ M or 1 μ l saline was then added for a further 10 min at 37°C. After the SNP incubation 50 μ l 3 $\times$ sample buffer containing the reducing agent, 2-mercaptoethanol and incubated for 5 min at 95°C. Platelet lysates were stored at -70°C with protein concentration determined by RC/DC protein assay. After the -70°C-stored samples were rapidly thawed in a 37°C water bath, an aliquot of 30 μ g protein were run on a 4-20% sodium dodecyl sulfate-polyacrylamide gel using a Bio-Rad miniblott electrophoresis and protein transfer systems. The fluorescence intensity of the P-VASP (ser 157), P-VASP (ser 239), cGKI, P-Selectin and VASP protein bands were quantified by multi-channel densitometry using Bio-Rad VersaDoc MP 4000 imager and Quantity One imaging software.

Statistical Analysis

Data are expressed as mean $\pm$ SEM (standard error of the mean). Comparison between the DBHD/PLGA, SNAP polymer coating effects and control groups were analyzed by the one-way ANOVA with a multiple comparison of means using Student's *t*-test. All statistical analyses were performed using the statistical program SAS JMP (SAS Institute Cary, NC). Values of $P < 0.05$ were considered statistically significant for all tests.

3. Results

3.1. NO Effects from DBHD/N₂O₂ and SNAP-containing ECC in RT Model

The hemodynamic parameters such as heart rate, pO₂, pCO₂ and pH remained near normal values during the 4 h experimental period. Mean arterial pressure (MAP) decreased over the 4 h blood exposure to the DBHD/N₂O₂ and SNAP-containing ECC compared to baseline (48 $\pm$ 3, $n=5$ and 37 $\pm$ 2, $n=4$ versus 69 $\pm$ 3, $n=18$, respectively) but maintenance isotonic fluids were infused to help maintain MAP.

To ascertain the differential formation of thrombus in the thrombogenicity chamber (i.e., the 3/8 inch ID Tygon tubing 8 cm in length) of the DBHD/N₂O₂, SNAP, PVC control and E2As control polymer-coated ECC, 2-dimensional (2D) image analysis was performed after

4 h of blood exposure. These thrombi area measurements were quantitated and, as shown in Figure 2, the thrombus area of the DBHD/N₂O₂ and SNAP polymer ECC were significantly smaller when compared to the PVC and E2As control polymer ECCs.

3.2. Effects of DBHD/N₂O₂, SNAP and Control Polymers on Rabbit Platelet Function and Surface Receptor Expressions

Platelet function during exposure to the DBHD/N₂O₂, SNAP, E2As control or PVC control polymer-coated ECC was assessed by observing platelet aggregation (Figure 3A), the level of collagen-stimulated P-selectin (Figure 3B) and the platelet fibrinogen receptor subunit, CD61 (or IIIa), expressions (Figure 3C). The percent of platelet functional aggregation, as determined by ex vivo collagen (25 µg/ml) stimulation of PRP, was measured by optical turbidity at a normalized platelet concentration of 2×10^8 platelets/ml. The ability of platelets to aggregate was only maintained with the DBHD/N₂O₂ or SNAP polymer ECCs whereas platelets from the E2As control or PVC control polymer-coated ECCs were significantly reduced in their ability to aggregate upon collagen stimulation at the 4 h time point (Figure 3A). In fact, the PVC control polymer-coated ECC reduced the platelet count below the level needed to normalize the platelet concentration and, thus, aggregation was not run at the 4 h time period.

To ascertain if the observed reduction in platelet aggregation with the E2As or PVC control polymers after 4 h blood exposure was due to direct effects on platelet activation, expression of the platelet membrane adhesion glycoprotein, P-selectin (CD62P), was measured by fluorescence-activated cell sorting (FACS) analysis. When platelets become activated, the surface expression of P-selectin increases [22]. As shown in Figure 3B, the collagen (40 µg/ml)-stimulated P-selectin expression was statistically unchanged after 4 h for all experimental groups but the PVC control ECC group showed a trend to decrease in collagen-stimulated P-selectin expression as compared to the baseline (no ECC present). The collagen-stimulated P-selectin expression for the 4 h time point for the PVC control ECC had an increase in P-selectin of 0.61 ± 2 mean fluorescence intensity (MFI) while the DBHD/N₂O₂, E2As control polymer ECC and no ECC baseline were increased 2.34 ± 1 , 4.40 ± 2 and 4.27 ± 1 MFI, respectively.

In order to understand if the DBHD/N₂O₂, E2As control or PVC control polymer ECCs can induce changes in the IIb/IIIa (fibrinogen) receptor, a specific antibody to IIIa (CD61) was used to evaluate platelet surface expression of IIb/IIIa via flow cytometry. As shown in Figure 3C, the platelet IIIa surface expression after 4 h for DBHD/N₂O₂ and E2As control ECC groups was unchanged compared to the no ECC baseline levels. The IIIa expression after 40 µg/ml exogenous collagen stimulation, however, significantly decreased after 4 h for PVC control ECC group compared to no ECC baseline.

3.3. Effects of NO releasing Polymers on Platelet P-VASP (ser 157), P-VASP (ser 239) and cGKIβ Expression

In order to determine if the NO released from DBHD/N₂O₂ or SNAP polymer ECCs would maintain rabbit platelet quiescence but still allow normal stimulation by exogenous NO with the NO donor, SNP, expressions of platelet P-VASP (ser 157), P-VASP (ser 239) and cGKI were investigated for changes in the NO/cGMP signaling pathway by Western immunoblot assays. In comparison, human platelet responses to NO/cGMP or cAMP pathway mediators have shown that P-VASP (ser 157) is associated with upregulation of cAMP pathway while the P-VASP (ser 239) is linked more to intracellular cGMP signaling, however, crosstalk does exist between these two signaling paths [20, 23, 24]. As shown in Figures 3 and 4, rabbit platelet expression of the selective phosphorylation of P-VASP (ser 157) and P-VASP (ser 239), respectively, is nearly wholly dependent on the phosphorylation

of serine 239 (Figure 4A) compared to lack of P-VASP (ser 157) expression (Figure 3A). Densitometric analysis of the western blots shows that the ratio of P-VASP (ser 157)/unphosphorylated VASP (Figure 3B) essentially shows little increase in this ratio over baseline (0.24 ± 0.09) due to NO release from NO containing polymers with or without further NO stimulation using $100 \mu\text{M}$ SNP. In contrast, rabbit platelets in response to NO released from DBHD/ N_2O_2 or SNAP polymer-coated ECC showed that when NO levels were elevated by the DBHD/ N_2O_2 polymer or by the exogenous NO donor, SNP, the ratio of P-VASP (ser 239)/VASP expression was upregulated 2 to 3.7 fold over baseline. As shown in Figure 4B, after 3 h of blood exposure to the DBHD/ N_2O_2 polymer ECC, the P-VASP (ser 239)/VASP ratio was 0.44 ± 0.16 (2.2 fold increase) compared to baseline (0.2 ± 0.08 ; NS). Significant increase, however, was observed with platelets from the 3 h exposure sample when further stimulated with $100 \mu\text{M}$ SNP over baseline (0.74 ± 0.33 vs 0.2 ± 0.08 ; $p < 0.05$, $n=5$). The rabbit platelet P-VASP (ser 239)/VASP ratio after 4 h exposure to NO releasing polymers had a reduced expression compared to the 3 h exposure but still showed a 2-3 fold increase over baseline. At this point the question was asked if the increase in the P-VASP (ser 239)/VASP ratio was linked to increases in the cGMP-dependent protein kinase, cGKI .

Rabbit platelet cGMP-dependent protein kinase, cGKI , expression was evaluated via western blotting and then quantified by fluorescence densitometry after 2, 3 and 4 h exposure to DBHD/ N_2O_2 , SNAP and control polymer ECCs (Figure 5). As far as the expression of the cGMP pathway intermediate protein kinase, cGKI , which uses VASP as a substrate, only the 4 h exposure to E2As control polymer ECC showed significant increases in cGKI and the SNAP-containing polymer ECC shows a trend to higher cGKI levels.

3.4. In Vitro Effects of cGMP Pathway Mediators on Platelet P-VASP (ser 157), P-VASP (ser 239) and cGKI β Expression

In order to better understand the in vivo results on the NO/cGMP signaling pathway responses in rabbit platelets we need to know if the rabbit NO/cGMP pathway is regulated similarly as shown previously in human platelets [23, 25]. The effects of the soluble guanylyl cyclase (sGC) inhibitor, ODQ, and the phosphodiesterase V (PDE V) inhibitor, dipyridamole, which increases levels of intracellular cGMP were first evaluated on rabbit platelet aggregation with or without the NO donor, SNP, using optical turbidity, B and C). In washed platelets from rabbits, $20 \mu\text{M}$ ODQ was able to attenuate the inhibitory effects of 10 (Figure 6B) and $100 \mu\text{M}$ SNP (Figure 6C) on aggregation and show unchanged aggregation responses compared to the DMSO vehicle response (Figure 6A and 6D). In contrast, SNP alone or in presence of $10 \mu\text{M}$ dipyridamole showed a concentration-dependent reduction in rabbit platelet aggregation compared to the saline controls (Figure 6D). As expected with the enhancer of cGMP levels, dipyridamole, there was a significant 1.8 fold decrease over the saline control in % aggregation (39.8 ± 7.5 vs 72.0 ± 15.8 , $p < 0.05$, $n=4-5$). In addition, increasing levels of SNP exacerbated the dipyridamole anti-aggregatory effects compared to saline control (Figure 6D). Knowing that ODQ and dipyridamole mediate the NO/cGMP pathway and their effects on platelet aggregation, it is important to show that the cGMP signaling proteins are also affected, in particular, cGKI , P-VASP (ser 239) and P-VASP (ser 157) by ODQ and dipyridamole as in human platelets.

Shown in Figure 7A, rabbit washed platelets responded to SNP in a concentration dependent fashion by increasing cGKI and $20 \mu\text{M}$ ODQ decreasing the protein. Only $100 \mu\text{M}$ SNP alone showed an increase in P-VASP (ser 157). The question at this point is whether the antibody for P-VASP (ser 157) crossreacts with rabbit P-VASP (ser 157). Figure 7C shows clearly that the antibody does recognize this phosphorylated protein at serine 157. Based on the fact that the antibody for P-VASP (ser 157) is functional, we found that rabbit P-VASP

(ser 157) is not directly upregulated by a cAMP agonist, PGE1 (10 μ M) (Figure 7B), while in human platelets, P-VASP (ser 157) is markedly upregulated by PGE1 (Figure 7D).

The effects of cGMP pathway manipulation by ODQ and dipyridamole on P-VASP (ser 239) (figure 8A) shows that the ratio of P-VASP (ser 239)/VASP is upregulated by 10 and 100 μ M SNP but was significantly increased by 100 μ M SNP in the presence of 10 μ M dipyridamole. In contrast, SNP had no effect on the ratio of P-VASP (ser 157)/VASP and dipyridamole failed to further increase the phosphorylated form of P-VASP (ser 157) (Figure 8B). Rabbit platelets also showed no increase in P-VASP (ser 239) (Figure 8C) or in P-VASP (ser 157) (Figure 8D) to 10 μ M PGE1.

4. Discussion

In vivo testing NO releasing polymer coatings (i.e., the NO donor, DBHD/PLGA, and the RSNO, SNAP, polymers) in an extracorporeal circuit has demonstrated for the first time that two mechanistically-different polymers can release similar NO levels and mediate, in part, platelet quiescence through the upregulation of the NO/cGMP signaling pathway. Specifically, the upregulation of cGMP protein kinase-dependent VASP (ser 239) phosphorylation after 4 h of blood exposure in a rabbit model of thrombogenicity was observed. This work additionally shows that the two different NO-releasing polymers significantly reduced ECC thrombus formation and preserved normal platelet aggregation compared to their respective non-NO releasing controls. The ability for the circulating rabbit platelet to maintain normal function as measured via ex vivo aggregation shows that the NO released from the polymers is sufficient to maintain or upregulate the cGMP pathway, as indicated by the increase in P-VASP (ser 239), to a point that does not inhibit platelet aggregation. The NO released from surface polymers appears to locally maintain platelet integrity and thus mimic the physiological role of NO from the vascular endothelium. The normal endothelium has been shown to release NO at a flux rate of $0.5 - 4 \times 10^{-10}$ mol $\text{cm}^{-2}\text{min}^{-1}$ and represents normal local release of NO [26]. In our work the NO flux rates for the DBHD/N₂O₂ and SNAP-containing polymer ECC were initially on average 10×10^{-10} mol $\text{cm}^{-2}\text{min}^{-1}$ and 2×10^{-10} mol $\text{cm}^{-2}\text{min}^{-1}$, respectively, and after 4 h of blood exposure the NO flux rates remained at least 75% of the initial flux rates. Even in face of the strong contact activation of prothrombotic processes elicited by the ECC surface, these ECC NO flux rates maintained ECC patency through marked attenuation of ECC thrombus formation and sustained the rabbit platelets in a quiescent state through predominantly the cGMP signaling pathway and not the cAMP path as shown both *in vivo* and *in vitro*. Understanding how platelets in this RT model behave to various treatments of antiplatelet interventions such as ECC surface release of NO will provide realistic insight into how these treatments may provide antiplatelet effects in the clinical situation.

Extensive research exists demonstrating that NO mediates the resting state in human platelet by the upregulation of cGMP signaling pathway and increased phosphorylation of both serine 239 and 157 in VASP [17, 20, 23], the mechanism of NO mediation of platelet quiescence in the rabbit model of thrombogenicity has not been investigated. Previous work, including ours, has established that NO releasing polymers (NORel) can prevent loss of circulating platelets and monocytes in the RT model and limited clinical ECLS [6, 27, 28]. These experiments demonstrate significant evidence for the importance of NO in defining, in part, the hemocompatibility of biomaterials used in ECLS and mimicking NO released from normal functioning vascular endothelium. The intracellular signaling pathway involved in mediating the NO effects on these rabbit platelet responses in this thrombogenic model primarily involves the upregulation of the cGMP-dependent protein kinase, cGKI, and subsequent elevation of the phosphorylation of its established substrate, the focal adhesion protein VASP. The phosphorylation of the serine 239 site (P-VASP (ser 239)) of this rabbit

platelet intracellular cGMP-dependent protein was mainly upregulated over the other phosphorylation site, serine 157 (P-VASP (ser 157)). Relevance of this P-VASP (ser 239) increase in rabbit platelet upon NO stimulation shows that what is known in human platelet cGMP/cAMP signaling is also present in the rabbit platelet signaling and that the rabbit model can be used to monitor NOrel polymer effects in mediating P-VASP (ser 239) and maintain platelet quiescence. Previous work using human platelets has demonstrated that VASP and VASP phosphorylation (P-VASP) is a useful biomarker of cGMP signaling and therefore the NO effects in human platelets [20, 29]. Much of this research has established P-VASP involvement in the inhibition of agonist-induced platelet aggregation and, specifically, the activation of the platelet fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$ [30, 31]. The best method thus far in analyzing the cGMP signaling upregulation and cGKI activity has been the use of 16C2 monoclonal antibody to P-VASP (ser 239) shown to be specific to the cGMP-dependent pathway [20]. The 5C6 monoclonal antibody to P-VASP (ser 157) has been shown to analyze the cAMP specific activity in the human platelet [17]. Using these antibodies to P-VASP (ser 239 or 157), platelets after 4 h blood exposure to the NOrel polymer-coated ECCs showed significant upregulation of P-VASP (ser 239), but not P-VASP (ser 157), when ex vivo stimulated by SNP. Both P-VASP (ser 239) and P-VASP (ser 157) were slightly increased at 2, 3 and 4 h blood exposure on ECC (Figures 4B and 5B) but not to the point where the rabbit platelets were inhibited from aggregating when stimulated (Figure 2B). This level of NO release from either the DBHD/N₂O₂ or SNAP was sufficient to maintain platelets in the ECC in an inactive state even though there is strong stimulus for activation as seen in the non-NO releasing PVC ECC. It also appears that this level of released NO from these two polymers did not prevent the exogenous NO donor, SNP, from further upregulating the expression of P-VASP (ser 239) but not P-VASP (ser 157) indicating a cGMP pathway control of platelet quiescence rather than cAMP signaling. Other evidence of the normal function of the rabbit platelets after 4 h exposure on the ECCs is the expected increase in the membrane glycoprotein, P-selectin, and the α_3 subunit of the fibrinogen receptor (CD61) when ex vivo stimulated by collagen and measured by flow cytometry. The non-NO releasing PVC control ECC showed no collagen-induced P-selectin or CD61 due to maximal platelet activation prior to collagen. Further work is planned to investigate if greater NO release from polymers could actually inhibit platelet aggregation and significantly upregulate P-VASP (ser 239) even though this is not clinically relevant.

Much of the data from this work to this point indicates that P-VASP (ser 239) and, to a lesser degree, P-VASP (ser 157), mediates a continued resting state of rabbit platelets even when exposed for 4 h to NOrel polymer ECCs. However, no data exists that shows rabbit platelets actually responding to manipulators of cGMP or cAMP pathways. In vitro aggregometry data show (Figure 7) that SNP caused a decrease in platelet aggregation in a concentration-dependent fashion. The soluble guanylate cyclase inhibitor, ODQ, maintained platelet aggregation in the face of SNP near control levels while the phosphodiesterase V inhibitor, dipyridamole, which enhances cGMP activity, maximally inhibited platelet aggregation compared to control. These data indicate that rabbit platelets respond to SNP and the released NO act through an active cGMP-dependent pathway similarly to the response seen in human platelets [32]. In order to determine if the attenuation in rabbit platelet aggregation is concomitantly tied to an increase in P-VASP (ser 239) or P-VASP (ser 157), western analysis was performed on lysates of washed rabbit platelets. SNP (Figure 8) shows a trend to upregulate P-VASP (ser 239) but not P-VASP (ser 157). Dipyridamole significantly increased the 100 μ M SNP-induced expression of P-VASP (ser 239) two fold over the saline control. ODQ prevented any upregulation of P-VASP (ser 239). To check if P-VASP (ser 157) is upregulated in rabbit platelets, the cAMP agonist, PGE1, was shown not to increase P-VASP (ser 157) or P-VASP (ser 239). These data, therefore, may indicate that P-VASP (ser 239) is strongly involved in mediating the responses in rabbit platelets to the released NO from the NO donor, SNP. Aktas *et al.* have shown that the PDE V inhibitor

does increase human platelet cGMP activity and enhances the cGMP-dependent signaling as well as P-VASP (ser 239), but not P-VASP (ser 157), upregulation [23]. Our data in the rabbit platelet supports their data from the human platelet. More investigation is needed to ascertain if P-VASP (ser 157) is involved to any degree in maintaining, in part, rabbit platelet quiescence.

5. Conclusions

This study has demonstrated that NO released from ECC surface coatings, which release NO via two different processes, elicits rabbit platelet quiescence by the upregulation of the platelet NO/cGMP signaling pathway and, specifically, the phosphorylation of the serine 239 subunit of vasodilator-stimulated phosphoprotein. The release of NO from NO-generating polymer coatings preserves circulating platelet function, as measured by P-selectin and CD61 expressions as well as maintaining platelet aggregation. These results indicate that locally-generated NO from ECC surface polymer coatings can increase hemocompatibility of extracorporeal devices by upregulating the platelet quiescence mechanism via the NO/cGMP signaling pathway and, in particular, the phosphorylation of serine 239 of VASP. The level of P-VASP (ser 239) expression can serve as a biomarker of NO/cGMP pathway activity and provide benefit for the RT model as a screening protocol for any locally-released NO polymer. Studies investigating improvements in the NO-releasing polymer reduce ECC thrombogenicity as well as in the measurement of P-VASP (ser 239) using flow cytometry are planned for future studies. The measurement of P-VASP (ser 239) and use of the rabbit as a thrombosis model have the potential to provide an effective monitor of the NO/cGMP signaling needed to maintain platelet quiescence and effective hemocompatible surface testing.

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Abbreviations

sGC	(soluble guanylate cyclase)
P-VASP (ser 239)	(serine 239 phosphorylation of VASP)
P-VASP (ser 157)	(serine 157 phosphorylation of VASP)
cGKI	(cGMP-dependent protein kinase types I subunit)
NORel	(NO releasing)
RSNO	(<i>S</i> -nitrosothiol)
DBHD	(dibutylhexansdiamine)
SNP	(sodium nitroprusside)

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Figure 2.

NO effects on ECC thrombus formation, platelet aggregation and fluorescent-activated cell sorting (FACS) analysis for circulating platelet P-selectin (CD62P) and fibrinogen receptor IIIa subunit, CD61. (A) Thrombus formation of NO releasing and control polymer ECCs after 4 hours blood exposure in the rabbit thrombogenicity model. The data are means $\pm$ SEM. * = $p < 0.05$, PVC control ECC vs NORel/PLGA and SNAP/E2As ECCs after 4 h. (B) Time course of platelet aggregometry. Platelet rich plasma was prepared and the aggregation initiated by 10 μ g/ml collagen. The data are means $\pm$ SEM. * = $p < 0.05$, baseline vs E2As control after 4 h. PVC control ECC rabbits consumed significant platelets after 4 h and unable to run aggregation. (C) Collagen-induced (40 μ g/ml) platelet P-selectin mean fluorescence intensity (MFI) after 4 h on ECC in NO releasing and control polymers. (D) Collagen-induced (40 μ g/ml) platelet CD61 mean fluorescence intensity (MFI) after 4 h on ECC in NO releasing and control polymers. The data are means $\pm$ SEM. * = $p < 0.05$, collagen-induced CD61 baseline vs PVC control ECC.

A

B

Figure 3.

Effects of NO on P-VASP (ser 157), P-Selectin and VASP expressions from rabbit platelet lysates after 1, 2, 3 and 4 h blood exposure with NO releasing and control polymer ECCs. (A), Representative immunoblot: aliquots (30 μ g) of reduced lysate samples from various time periods on ECCs in NOGen or control polymers were separated on 4-20% polyacrylamide gels, protein transferred to poly(vinyl-difluoride) membranes and then immunostained with FITC conjugated mouse anti-human P-VASP (ser 157), goat anti-human P-Selectin (C-20) and rabbit anti-human VASP (H-90) antibodies. Molecular weight markers are shown on left with positions of the relevant P-VASP (Ser 157), P-Selectin and VASP proteins. (B) Western blot of lysate samples in (A) were quantified by multi-channel densitometry using imaging software and digital imager. The ratio of P-VASP (ser 157) over unphosphorylated VASP expressions was calculated. The data are means $\pm$ SEM.

A

B

Figure 4.

NO effects on P-VASP (ser 239), cGK1 and VASP expressions from rabbit platelet lysates after 1, 2, 3 and 4 h blood exposure with NO releasing and control polymer ECCs. (A), Representative immunoblot: After SDS-PAGE electrophoresis and electroblotted blots were immunostained with FITC conjugated mouse anti-human P-VASP (ser 239), goat anti-human cGK1 (L-16) and rabbit anti-human VASP (H-90) antibodies. Molecular weight markers are shown on left with positions of the relevant P-VASP (ser 239), cGK1 and VASP proteins. (B) Western blot of lysate samples in (A) were quantified by multi-channel densitometry using a digital imager. The ratio of P-VASP (ser 239) over unphosphorylated VASP expressions was calculated. The data are means $\pm$ SEM. * = $p < 0.05$, baseline vs baseline + SNP, 2 and 3 h DBHD/N₂O₂ containing ECC + sodium nitroprusside (SNP).

Figure 5.

Time-dependent effects of NO-releasing ECCs on the cGMP-dependent protein kinase type II expression in rabbit platelet lysates. The data are means $\pm$ SEM. (A):* = $p < 0.05$, baseline vs 3 and 4 h PVC and E2As control ECCs. (B):* = $p < 0.05$, baseline vs 4 h E2As control ECC + SNP.

Figure 6.

Effects of NO/cGMP pathway mediators on normal rabbit platelets using aggregometry. Platelet rich plasma was prepared and the aggregation initiated by 25 $\mu\text{g/ml}$ collagen. Prior to collagen stimulation, 4% DMSO vehicle, 20 μM 1H-[1,2,4]-oxadiazolo [4, 3-a]-quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor, or 10 μM dipyridamole were added to rabbit platelets for 10 min. After this incubation, 0 (A), 10 (B) or 100 μM (C) sodium nitroprusside (SNP; NO donor) was then added for 10 min. (D) The maximum % aggregation was determined for each compound combination and graphically represented. The data are means $\pm$ SEM. * = $p < 0.05$, DMSO control vs DMSO control + 10 and 100 μM SNP and 10 μM dipyridamole with and without SNP.

Figure 7.

Effects of NO/cGMP pathway mediators on normal washed rabbit (A & B) and human (C & D) platelet expression of P-VASP (ser 157). P-VASP (ser 239). P-selectin, cGK1 and VASP proteins as assessed via western blotting. The human platelet response to NO donors confirms that the antibody for P-VASP (ser 157) detects this phosphoprotein markedly while not as active in NO-stimulated rabbit platelets (A & B). Also it is shown that the cAMP agonist, PGE1, upregulates P-VASP (ser 157) in human platelets (D) but not in rabbit (C). PGE1-upregulated P-Selectin or cGK1 expression in rabbit platelets (B). In human platelets, 10 μ M PGE1 markedly upregulates P-selectin, cGK1 and P-VASP (ser 157) expressions.

Figure 8.

Effects of NO/cGMP pathway mediators on SNP-induced P-VASP (ser 157) and P-VASP (ser 239) expressions in washed rabbit platelets. (A), Ratio of P-VASP (ser 239)/VASP expressions after 0, 10 or 100 μ M SNP in rabbit washed platelet lysate with or without 20 μ M ODQ or 10 μ M dipyridamole. (B) Ratio of P-VASP (ser 157)/VASP expressions after 0, 10 or 100 μ M SNP in rabbit washed platelet lysate. The data are means $\pm$ SEM. * = $p < 0.05$, DMSO control vs 10 μ M dipyridamole + 100 μ M SNP.